African Journal of Biotechnology Vol. 10(51), pp. 10387-10392, 7 September, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.187
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

A comparative study of ATPase subunit 9 (*Atp9*) gene between cytoplasmic male sterile line and its maintainer line in soybeans

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Accepted 4 July, 2011

ATPase subunit 9 gene (*Atp9*) is an important functional gene in mitochondria, and is closely related with energy supply. RNA editing of *atp9* gene was associated with male sterility in plants. In this study, the *atp9* gene in soybeans was cloned from a soybean cytoplasmic male sterile line NJCMS2A and its maintainer line NJCMS2B. Sequence alignment was performed, and protein structures were analyzed and compared between the soybean cytoplasmic male sterile line NJCMS2A and its maintainer line NJCMS2B. The results show that the fragments with identical sequences of *atp9* gene were amplified from the genomic DNA of NJCMS2A and NJCMS2B, while the sequences of *atp9* were different when they were amplified from cDNAs of NJCMS2A and NJCMS2B. RNA editing of *atp9* gene in the maintainer line NJCMS2B was detected with two nucleotide sites (C to U) in the conserved region, leading to conversion of hydrophilic amino acid serine into hydrophobic leucine. No RNA editing was detected in *atp9* gene in the male sterile line NJCMS2A. The putative trans-membrane structures of the atp9 proteins were different, and their trans-membrane directions were opposite.

Key words: Soybean, cytoplasmic male sterility, *atp9* gene, RNA editing.

INTRODUCTION

F_0F_1-ATPase complex of mitochondrial inner membrane, which consisted of the proton channel F_0 factor (*atpA*) and the F_1 factor *(atp6 and atp9*) catalyzing the ATP synthesis, plays an important role in the energy formation in plants. Gene loci changes of *atp6* and *atp9* subunit or their upstream or downstream DNA sequence changes were closely related to plant male sterility, and they directly affected the energy supply in a certain period or a certain organ, thereby leading to male sterility by influencing growth and development (Kadowaki and Harada, 1989; Akagi et al., 1994).

The RNA editing refers to the molecular processes in which the genetic information of DNA is altered through mRNA base insertion, deletion or replacement after gene transcription, thus resulting in changes of amino acid sequence and encoded protein. RNA editing expands the genetic information, thus making the environment more adaptable to the organisms. This phenomenon was first reported in mitochondria of *Trypanosoma brucei* with four non-DNA-encoded U residues in the COX2 transcript (Benne et al., 1986). Later, the non-DNA-encoding was found in chloroplasts and mitochondria in higher plants, fungi mitochondria, animal cells and viruses, etc (Araya et al., 1992, 1998). The common type of RNA editing in plant organelle was conversion of C to U. RNA editing could cause nucleotide changes, thereby resulting in amino acid alterations. Therefore, RNA editing played an important role in the normal mitochondrial function (Iwabuchi et al., 1993; Mulligan et al., 1999). Wei et al. (2008) studied the RNA editing of *atp9* gene in Yunnan HL type male sterile rice, and then speculated that RNA editing of *atp9* gene may be associated with rice cytoplasmic male sterility. In another report, cytoplasmic male sterility was induced after transferring unedited *atp9* gene into fertile tobacco, and this confirmed the correlation between the RNA editing of *atp9* gene and tobacco cytoplasmic male sterility (Hernould et al., 1993).

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In 1990, *atp9* gene sequence was first reported by Grabau et al. (1990) in soybean mitochondria. However, Brown et al. (1991) and Pesce and Grabau (1993) reported RNA editing of mitochondrial *atp9* gene of soybean Williams82, respectively. They found C-to-U changes at position 20 and 50 in the nucleotide sequence after gene transcription. Up to now, there was no report about the relation between RNA editing of *atp9* gene and soybean male sterility. CMS (cytoplasmic male sterility) plays an important role in the utilization of crop heterosis, moreover, it is of important significance on theory and practice to study the genetic base and mechanism of CMS. To reveal the molecular mechanism of soybean cytoplasmic male sterility, in this study, soybean cytoplasmic male sterile line NJCMS2A and its maintainer line NJCMS2B were used as materials to investigate the relation between RNA editing of *atp9* gene and soybean cytoplasmic male sterility.

MATERIALS AND METHODS

Soybean CMS (cytoplasmic male sterility) line NJCMS2A and its maintainer line NJCMS2B were used in this study. Soybean CMS line NJCMS2A was developed through consecutive backcross procedures with the F_2 male sterile plants of N8855 x N1628 cross as donor parent and N1628 (designated as NJCMS2B afterwards) as recurrent parent (Ding et al., 1998, 2002; Bai and Gai, 2003, 2006).

The CMS line NJCMS2A and its maintainer line NJCMS2B were grown at the Jiangpu Experimental Station of National Center for Soybean Improvement (NCSI) in Nanjing Agricultural University in the summers of 2008 and 2009. The leaves and flower buds were collected from NJCMS2A and NJCMS2B, respectively. These samples were quickly frozen in liquid nitrogen, and stored in the refrigerator at -80℃ prior to use.

Extraction of genomic DNA

Extraction of genomic DNA in leaves was performed with the CTAB method (Wang and Fang, 2003).

RNA extraction and reverse transcription

Total RNA in flower buds was extracted using RNAsimple Total RNA Kit [Tiangen Biotech (Beijing) Co., Ltd.] following the manufacturer's instruction. The strand cDNA was synthesized using Rever Tra Ace RT-PCR kit [Toyobo Biotech (Shanghai) Co., Ltd.] following the manufacturer's instruction.

Primer design

Specific primers *atp9*-F/R were designed with primer premier 5.0 (its copyright is by PREMIER Biosoft International) based on the *atp9* gene sequence (Accession Number: L17319.1) of the soybean Williams82 reported in NCBI (http://blast.ncbi.nlm. nih.gov/ Blast.cgi). The primers were synthesized by Invitrogen Biotech (Shanghai) Co., Ltd. according to the following sequences: *atp9*-F, 5'–GCGTGACGAGACTTTTTAT-3' ; *atp9*-R, 5' –CTACACCTTT CAAAAGGA GA-3'.

Recovery, cloning and sequencing of target fragments

Genomic DNA and cDNA from NJCMS2A and NJCMS2B were used as templates for PCR amplification respectively. PCR products were detected by electrophoresis on a 2.0% agarose gel. Then the target fragments were recovered and ligated to the pMD19-T vector [Takara Biotech (Dalian) Co., Ltd.] following the manufacturer's instruction. The ligation products were transformed into *E. coli* strain TOP10 (Tiangen Biotech (Beijing) Co., Ltd.). DNA samples were delivered to Invitrogen Biotech (Shanghai) Co., Ltd. for sequencing after extraction of plasmids and fragment size detection.

Sequence analysis

Sequence analysis of the target fragment was performed by BLAST (http:/blast.ncbi.nlm.nih.gov/ Blast.cgi) and ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/). Molecular weight and isoelectric point (pI) of the protein was predicted by ProtParam program (http://www.expasy.org_cgi-bin_protparam). The protein trans-membrane structure was predicted by TMHMM Server v.2.0 software (http://www.cbs.dtu.dk/ services/ TMHMM/) and displayed by Photoshop software (http://labs.adobe.com/ technologies/photoshop/). Amino acid sequence alignment was performed with clustalx1.81 software (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/), and the results were displayed by GeneDOC software (copyrighted by the Regents of the University of California).

RESULTS AND DISCUSSION

Detection of total RNA

The detection results of total RNA showed that bands of 28 S rRNA and 18 S rRNA were clear, while the results of UV spectrophotometer determination showed that A260/A280 rate was about 2.0, indicating that the extracted total RNA was of high purity and good quality.

Cloning of *atp9* gene

Atp9 gene was amplified using the genomic DNA of leaves and cDNA of flower buds from NJCMS2A and NJCMS2B as templates, respectively. A 300bp fragment can be amplified from the four samples (Figure 1), after which the four 300bp fragments were recovered and ligated to the pMD19-T vector, respectively. Positive clones identified by blue/white spots screening and PCR validation were delivered for sequencing (five independent clones for each sample).

Sequence analysis

The sequencing results of four samples were 276bp after the removal of vector sequences. ORF was identified as 222bp long, encoding 74 amino acids. However, there were two kinds of sequencing results: (1) Two cytosines (C) were identified at position 20 and 50 in NJCMS2A-

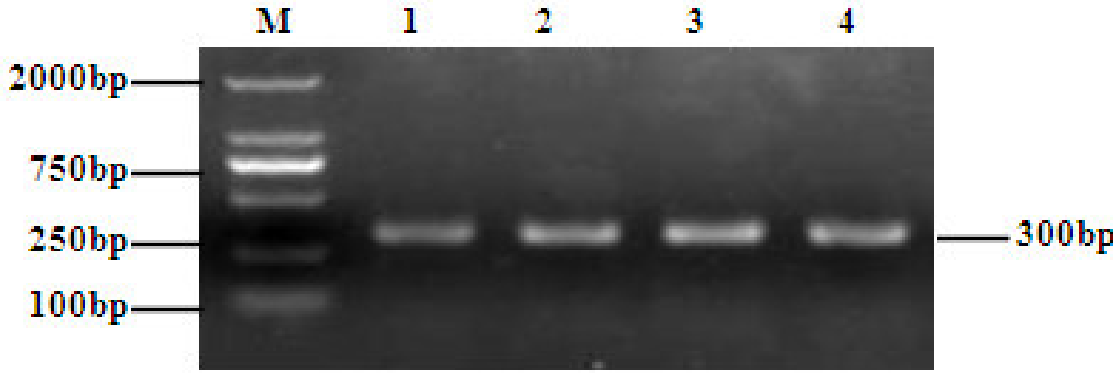

Figure 1. Amplification results of *atp9* gene from DNA and cDNA of NJCMS2A and NJCMS2B, respectively. M: Marker DL2000; 1: NJCMS2A-DNA; 2: NJCMS2B-DNA; 3: NJCMS2A-cDNA; 4: NJCMS2B-cDNA.

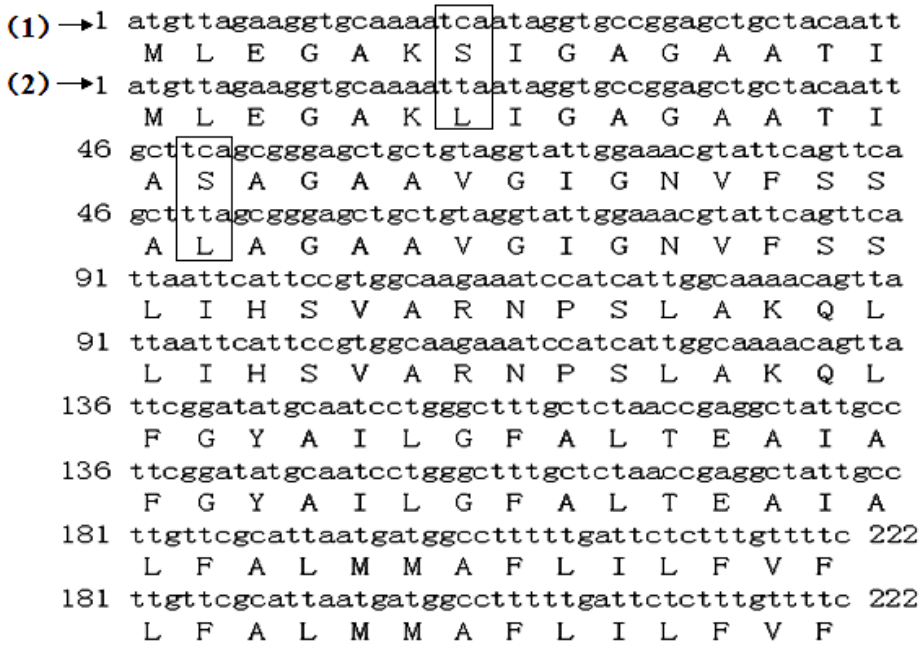

Figure 2. Comparison between two *atp9* gene sequencing results.

DNA, NJCMS2B-DNA and NJCMS2A-cDNA nucleotide, and the corresponding amino acids at position 7 and 17 were serines (S). (2) Two thymines (T) were identified at position 20 and 50 in NJCMS2B-cDNA nucleotide, and the corresponding amino acids at position 7 and 17 were leucines (L) (Figure 2).

The four sequencing results were translated into amino acid sequences. Amino acid sequence alignment was performed with clustalx1.81 software, and the results were displayed by GeneDOC software. *Atp9* gene of NJCMS2A-DNA and NJCMS2A-cDNA showed the same sequence with no RNA editing, while that of NJCMS2B-DNA and NJCMS2B-cDNA showed different sequences with RNA editing, namely the amino acids at position 7 and 17 of NJCMS2B-cDNA which changed from -S-S- into-L-L- (Figure 3).

Protein trans-membrane structure prediction

Atp9 gene encoded a protein of 74 amino acids with a molecular weight of 7.51kD and *pI* of 9.26 according to ProtParam analysis. Protein trans-membrane structure was predicted by TMHMM Server v.2.0 software. The results show that large difference was detected between the atp9 protein trans-membrane structures in NJCMS2A and NJCMS2B as they were in opposite directions. The *atp9* protein trans-membrane direction in NJCMS2A was

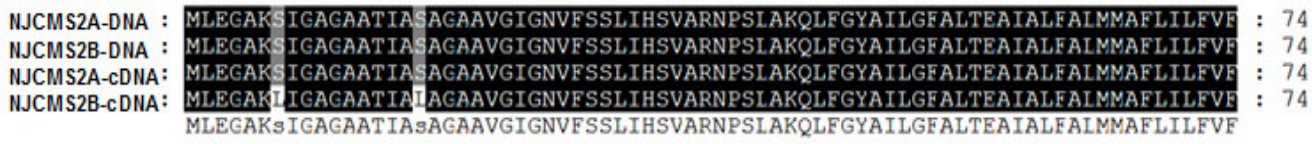

Figure 3. Amino acid sequence alignment profile of *atp9* gene.

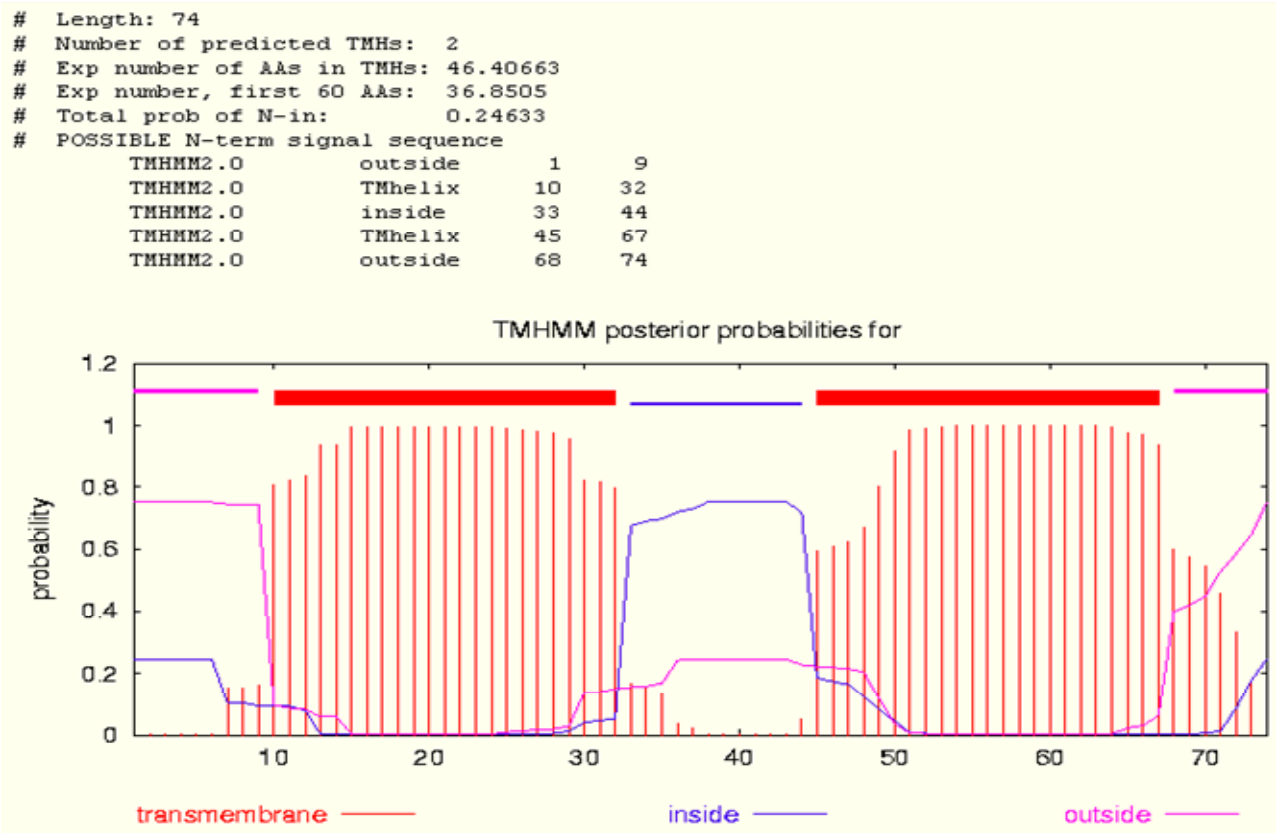

Figure 4. Protein trans-membrane structure prediction in NJCMS2A.

outer membrane - membrane - inner membrane - membrane - outer membrane (Figure 4), while in NJCMS2B, it was inner membrane - membrane - outer membrane - membrane - inner membrane (Figure 5).

To facilitate observation and comparison, the Photoshop software was used to change Figures 4 and 5 into the model diagram in Figure 6. According to Figure 6A, two trans-membrane domains were identified at position 10 to 32 and position 45 to 67 of atp9 protein structure in NJCMS2A, with a trans-membrane direction of outside - inside - outside; while in Figure 6B, two trans-membrane domains were identified at position 7 to 29 and position 49 to 71 of atp9 protein structure in NJCMS2B, with a trans-membrane direction of inside - outside - inside.

RNA editing commonly exists in most plants. The studies show that CMS was related with functional genes *atp6*, *atp9* and *cox2* in compound enzyme subunit involved in the respiratory metabolism of plants. These genes affected the growth and development of plants by influencing energy supply (Kadowaki et al., 1990; Dewey et al., 1991; Mohr et al., 1993; Song and Hedgcoth, 1994). Pollen abortion was induced after transforming an incompletely edited *atp9* gene in tobacco; however, normal fertility remained the same after transforming a completely edited *atp9* gene in tobacco (Hernould et al., 1993). Further study showed that the fertility of tobacco could be restored by inhibiting expression of the incompletely edited *atp9* gene (Zabaleta et al., 1996). In addition, CMS was obtained by transforming unedited *atp9* gene into tobacco (Araya et al., 1998). All these transgenic experiments confirmed the correlation between tobacco CMS and RNA editing directly. A comparative study, reported by Han et al. (2010), on RNA editing of *atp6* gene between CMS line NJCMS1A and

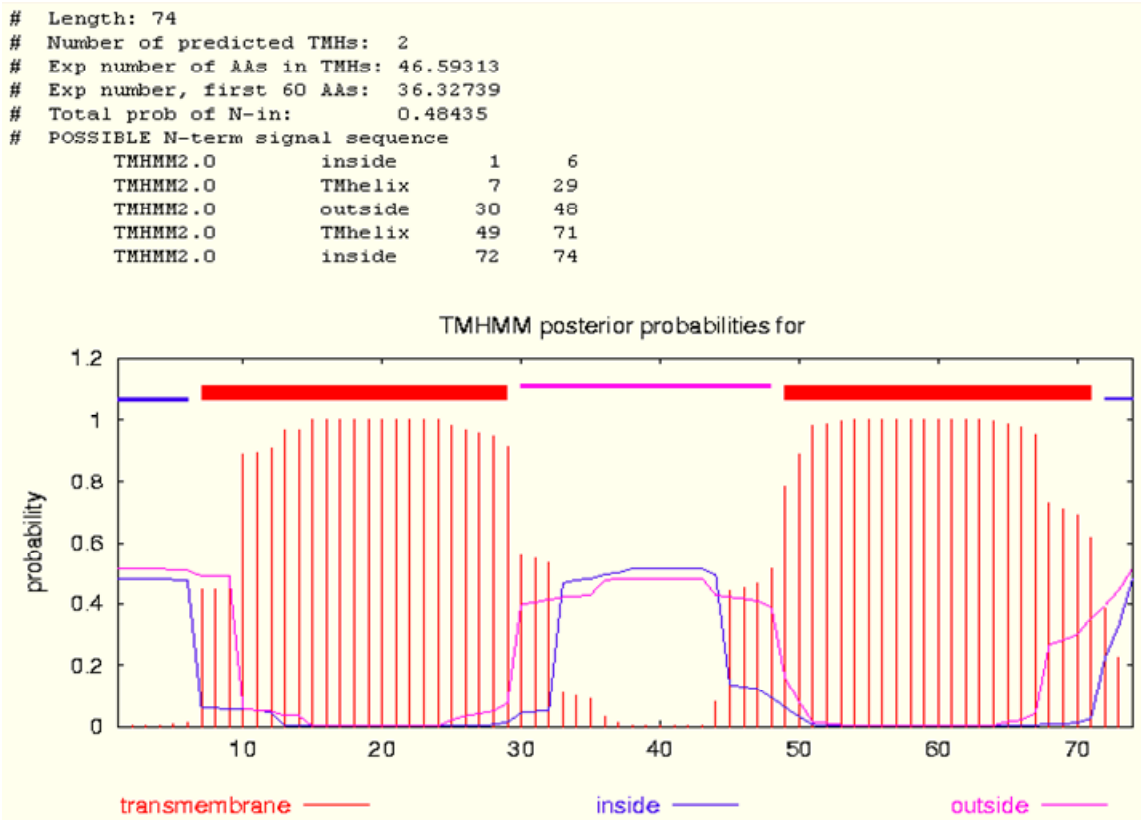

Figure 5. Protein trans-membrane structure prediction in NJCMS2B

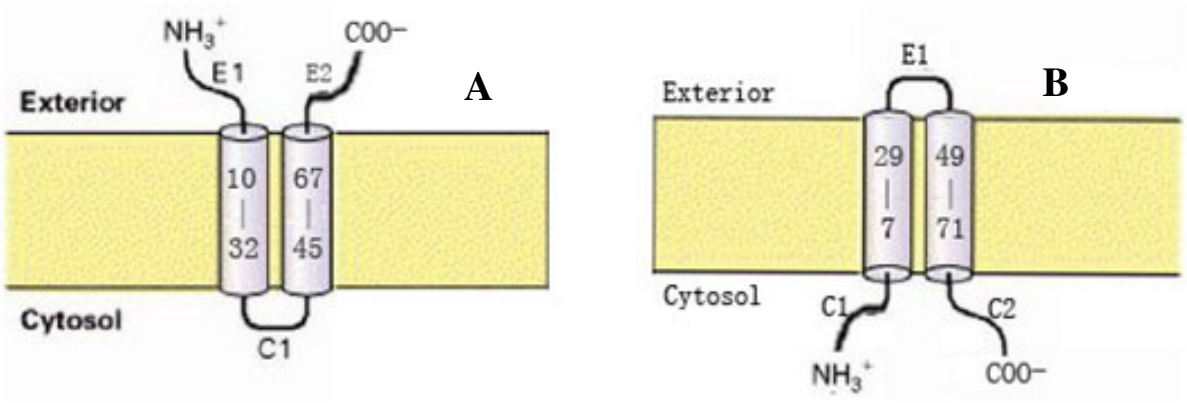

Figure 6. Trans-membrane structure model of atp9 protein. A: NJCMS2A; B: NJCMS2B.

maintainer line NJCMS1B in soybean showed different RNA editing sites in *atp6-3* gene between NJCMS1A and NJCMS1B, which may induce the structural and functional changes of ATP6 leading to NJCMS1A male sterility. In this study, cDNA and DNA sequence of *atp9* gene showed the same sequence as no RNA editing was accomplished, leading to male sterility in NJCMS2A, while in maintainer line NJCMS2B, RNA editing was ended by a conversion of C to U in cDNA transcription, leading to normal growth.

RNA editing in higher plants always occurred in protein coding regions of the transcripts. The editing site was the first or second site of codon mainly found with the conversion of C to U, which could induce the encoded amino acid changes, and improve the stability of transcripts and hydrophobicity of the encoded protein (Hanson et al., 1996). This study showed two C-to-U editing sites of *apt9* in the maintainer line NJCMS2B that led to a conversion of hydrophilic serine to hydrophobic leucine. Thus, the structures of trans-membrane protein

were changed with increasing protein stability. Normal fertility was demonstrated in NJCMS2B. Therefore, we concluded that male sterility was induced by dysfunction of ATP synthase affected by incomplete function of *apt9* resulting from none of the RNA editing in NJCMS2A. Busi et al. (2011) reported the mitochondrial dysfunction model by transforming unedited ATP synthase subunit 9 gene to *Arabidopsis thaliana.* This will be used to further study the relationship between unedited *atp9* gene and soybean cytoplasmic male sterility through transgene and transcriptome analysis.

ACKNOWLEDGEMENTS

The project was supported by the National High-Tech Research Program (863) (2009AA101106, 2011AA10A105) and the National Key Program for Transgenic Breeding (2008ZX08004-005).

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